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STUDIES ON DETOXICATION BY THE PERFUSED LIVER

I. HIPPURIC ACID SYNTHESIS AFTER IRRADIATION

II. BILIRUBIN CONJUGATION AND EXCRETION AFTER IRRADIATION

by

G.B. GERBER and J. REMY-DEFRAIGNE
(EURATOM) (C.E.N. - Mol)

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Studies on detoxication by the perfused liver
I. Hippuric acid synthesis after irradiation †

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(*Received* 6 *June* 1963 ; *revision received* 15 *July* 1963)

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An increase in synthesis of hippuric acid without addition of glycine was found in x-irradiated liver, indicating an expansion of the free glycine pool. On the other hand, synthesis of hippuric acid in x-irradiated liver was found to be slightly decreased when large amounts of glycine were present in the perfusate.

1. INTRODUCTION

Metabolic disorders of irradiated animals may be influenced or even caused by the ability of the body to detoxicate metabolites or foreign compounds.

A study of several of the more important reactions of detoxication after radiation in the isolated perfused liver has therefore been undertaken.

The latter system was selected, because it is sufficiently close to *in vivo* conditions while, at the same time, interference from starvation can be kept to a minimum.

Synthesis of hippuric and its analogues represents an important pathway of detoxication in rats as well as in humans (Williams 1956) and merits particular interest, since it has been shown that the glycine moiety of hippuric acid in irradiated rats is derived from a pool different in size and metabolic origin from that in non-irradiated rats (Gehrman, Lauenstein and Altman 1957, Lauenstein, Haberland, Hempelmann and Altman 1957, Gerber, Gerber, Altman and Hempelmann 1959).

2. METHODS

Male rats of the Wistar strain, weighing 250 to 300 g, which had been starved for 18 hours were used for all experiments. The group of irradiated rats was exposed to 1000 r whole-body x-irradiation (300 kv ; 100 r/min ; filter 2 mm Cu) and served as liver or blood donors for the liver perfusion 24 hours later.

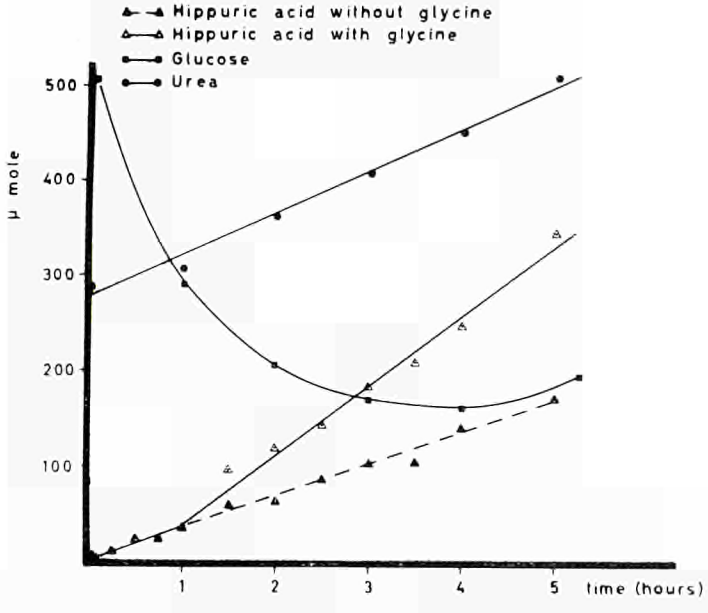
Perfusion of the isolated liver was carried out by a modification of the technique of Miller, Bly, Watson and Bale (1951), Green and Miller (1960), Gerber and Remy-Defraigne (in the press). The perfusate consisted of 30 to 35 ml of fresh heparinized rat blood to which 1 3 volume Ringer's solution and 150 mg of glucose had been added. Blood from normal rats was used for

† This work was carried out under Euratom contract 018–62–4B1AB. Dedicated to Professor B. Rajewsky for his 70th birthday.

perfusion of normal liver, and blood from x-irradiated rats for that of x-irradiated liver. Four hundred μmole of sodium benzoate in 5 ml of Ringer's solution were added to the perfusate as soon as the rate of blood-flow had reached a constant value (about 20 min after the start of the perfusion). One hour later, 400 μmole of glycine were added to the perfusate. Samples from the perfusate were taken, 5, 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min after the addition of the benzoate. Hippuric acid was isolated from the blood by descending paper chromatography of 200 μl serum on 5 cm-wide strips of Whatman No. 3 paper (ethanol H_2O, 80 20, v/v). The area containing hippuric acid and benzoic acid was localized under the ultra-violet lamp and dipped shortly into Altman's reagent (Gaffney, Schreier, Differrante and Altman 1954). Colour was developed for 5 min at 130°, the azlactone was eluted in 5 ml of methanol, and the optical density was determined at 458 mμ in a Zeiss spectrophotometer (Gaffney *et al.* 1954). Known amounts of hippuric acid were added to serum samples and served as reference for the calculation of the concentration of hippuric acid in the perfusate. Glucose and urea were also determined in deproteinized serum (Nelson 1944, Levine, Leon and Steigmann 1961).

3. Results

Synthesis of hippuric acid in the perfused liver as a function of time is shown in the figure. During the first hour, synthesis of hippuric acid is small and apparently limited by the amount of glycine available. After addition of glycine, synthesis of hippuric acid increases about four-fold and remains essentially constant during perfusion. If no glycine is added (broken line



Synthesis of hippuric acid, production of urea and utilization of glucose by the normal, perfused liver. 400 μmole of glycine were added to the perfusate after one hour in the experiment represented by the continuous line. No glycine was added in the experiment represented by the broken line.

	Number of livers perfused	Synthesis of hippuric acid (μmole/hour)		Synthesis of urea (μmole/hour)	Initial utilization of glucose† (μmole/hour)	Liver-weight (grams)
		Without addition of glycine	With addition of glycine			
Control	6	$2{\cdot}5 \pm 0{\cdot}5$‡	$9{\cdot}7 \pm 1{\cdot}2$‡	15	47	8·5
X-irradiated (1000 r)	5	$4{\cdot}2 \pm 0{\cdot}5$	$6{\cdot}1 \pm 0{\cdot}9$	14	50	8·3

†All values are expressed in μmole/hour per gram of liver. Only the initial decrease in glucose-concentration has been used for this computation, since concentration of glucose attains a stable value after two to three hours.

‡Standard deviation of the mean for the different experiments. The significance for the difference in the synthesis of hippuric acid is in both cases $P \leqslant 0{\cdot}015$.

Synthesis of hippuric acid in the isolated perfused liver of normal and x-irradiated rats.

in the figure), synthesis proceeds at the original low rate. Glucose concentration in the perfusate decreases initially and attains a stable value after two to three hours (see figure). Urea is produced during the total course of perfusion (see figure).

If normal and x-irradiated rats are compared (see table), one finds that synthesis of hippuric acid in irradiated liver is significantly higher during the first hour of perfusion when no glycine has been added, but that it is lower during the later time when the concentration of glycine is high. No difference exists, either in production of urea or initial utilization of glucose, between normal and x-irradiated rats.

4. Discussion

Synthesis of hippuric acid has been used by Arnstein and Neuberger (1951), to estimate the size of the ' free ' glycine pool (about 130 μmole per hour per 100 g). The ' free ' glycine pool of the perfused liver is, as may be expected, smaller (20 μmole liver), since only a part of the total-body free glycine is present in liver and perfusate. After glycine has been added to the perfusate, hippuric-acid synthesis proceeds at a rate comparable to that in the whole body, and this observation indicates that the perfused liver has retained its functional integrity.

The ' free ' glycine pool in the whole body is expanded after exposure to radiation (Gehrman *et al.* 1957, Lauenstein *et al.* 1957). This fact is also true for the isolated, perfused, irradiated liver, since more hippuric acid is synthetized in normal liver if no glycine is added. Such an expansion of ' free ' glycine pool can be due to a variety of factors, e.g. increased breakdown and decreased replacement of proteins, or derangement of other metabolic pathways. On the other hand, synthesis of hippuric acid under conditions of ample supply of glycine is slightly but significantly decreased after irradiation. It should, however, be pointed out that such a small decrease in enzyme concentration might not be very important under biological conditions, since the rate of synthesis of hippuric acid in the body is determined rather by the amount of available glycine than by the concentration of the enzyme or the metabolite to be coupled to glycine. Furthermore, the composition of irradiated liver differs in several respects from that of normal liver, and a comparison on the basis of liver-weight may not be entirely valid, particularly, if a small decrease in concentration of an enzyme is involved.

La synthèse d'acide hippurique a été déterminée avec et sans addition de glycine dans le foie isolé et perfusé de rats normaux et de rats irradiés par des rayons x.

On a trouvé une augmentation de la synthesè de l'acide hippurique sans addition de glycine dans le foie irradié, indiquant une expansion du pool de glycine libre.

D'autre part, on a trouvé que la synthèse de l'acide hippurique dans le foie irradié était légèrement diminuée après addition de glycine.

Die Hippursäuresynthese mit und ohne Zugabe von Glycin wurde in der isolierten perfundierten Leber normaler und bestrahlter Ratten bestimmt. Eine vermehrte Synthese von Hippursäure ohne Zugabe von Glycin, die nach Bestrahlung gefunden wurde, weist auf eine Vergrößerung des freien Glycin Pools hin. Andererseits war die Hippursäuresynthese der bestrahlten Leber etwas vermindert, wenn genug Glycin dem Perfusat zugegeben war.

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Studies on detoxication by the perfused liver
II. Bilirubin conjugation and excretion after irradiation†

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Formation of conjugated bilirubin and its excretion into the bile has been studied in the isolated perfused liver of normal and x-irradiated rats. Bilirubin is removed rapidly from the blood and is excreted, after a lag-period, to about 70–85 per cent as conjugated bilirubin into the bile. Conjugation occurs mainly with glucuronic acid but also with other compounds.

Removal of bilirubin from the blood and excretion of conjugated bilirubin in the bile are delayed in the perfused liver of x-irradiated rats.

1. Introduction

In the course of studies on detoxication by the isolated perfused liver of normal and x-irradiated rats, formation of hippuric acid from glycine and benzoic acid has been investigated (Gerber and Remy-Defraigne 1963 a, b). The present investigation is concerned with another important pathway of detoxication, the formation of glucuronide conjugates and their excretion into the bile.

2. Methods

The technique for perfusing the isolated rat liver was the one used previously (Miller, Bly, Watson and Bale 1951, Gerber and Remy-Defraigne 1963 b). The volume of the perfusate was 38–45 ml. Blood from x-irradiated rats was used to perfuse x-irradiated liver, and blood for normal rats to perfuse normal liver. Female rats, each weighing 200–300 g, were used in all experiments.

Whole-body x-irradiation (1000 r) of the liver and blood donors was carried out 24 hours before perfusion. All animals were starved for 18 hours before perfusion. After the flow of bile and perfusate had been stabilized (about 30 min after the start of perfusion) a solution containing 2·8–3·2 mg bilirubin was added to the perfusate. The bilirubin (Fluka) was dissolved in 0·5 ml 0·1 N NaOH, 2 ml of fresh rat serum were added, and the pH was adjusted to pH 7·6 with a solution of 0·15 M NaH_2PO_4. Samples were taken from the inflowing and from the outflowing perfusate during the course of the perfusion, and bile was collected over the time-periods indicated in figure 2.

Total bilirubin in the serum was determined by a modification of the method of Jendrassik and Cleghorn (1937). Free and conjugated bilirubin (as bilirubin diglucuronide) in blood and bile were measured according to the method of Weber and Schalm (1962). If haemolysis of the serum interfered with the determination of the free bilirubin, 0·5 ml of water was added to the chloroform

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extract after colour development. The mixture was then centrifuged, and the concentration of the azobilirubin in the upper phase was measured. The interfering colour from haemolysis remained in the chloroform layer.

Since it has been demonstrated recently (Isselbacher and McCarthy 1959), that conjugation of bilirubin takes place not only with glucuronic acid, but also with sulphate and other compounds, non-glucuronide-conjugated bilirubin was determined in a few bile samples after incubation with β-glucuronidase (Isselbacher and McCarthy 1959) and after hydrolysis in 0·1 N NaOH (Cole, Lathe and Billing 1956). The quantity of such non-glucuronide-conjugated bilirubin, as determined by incubation with β-glucuronidase, was 25–35 per cent and the amount of alkali-stable bilirubin was 15–20 per cent of the total conjugated bilirubin. The finding is in agreement with the observations of Isselbacher and McCarthy (1959).

3. Results and Discussion

Free bilirubin is removed rapidly from the blood after addition of bilirubin (during one passage through the liver about 10 per cent of the amount present in the perfusate) (figure 1). Maximal removal of bilirubin from the blood takes place 15 to 30 min after addition of bilirubin and diminishes progressively as the concentration of bilirubin in the perfusate decreases. Conjugated bilirubin is secreted into the perfusate in small quantities initially and its concentration is higher in the outflowing than in the inflowing perfusate. However, the concentration of conjugated bilirubin in the perfusate decreases quickly and only traces can be found after 30 min.

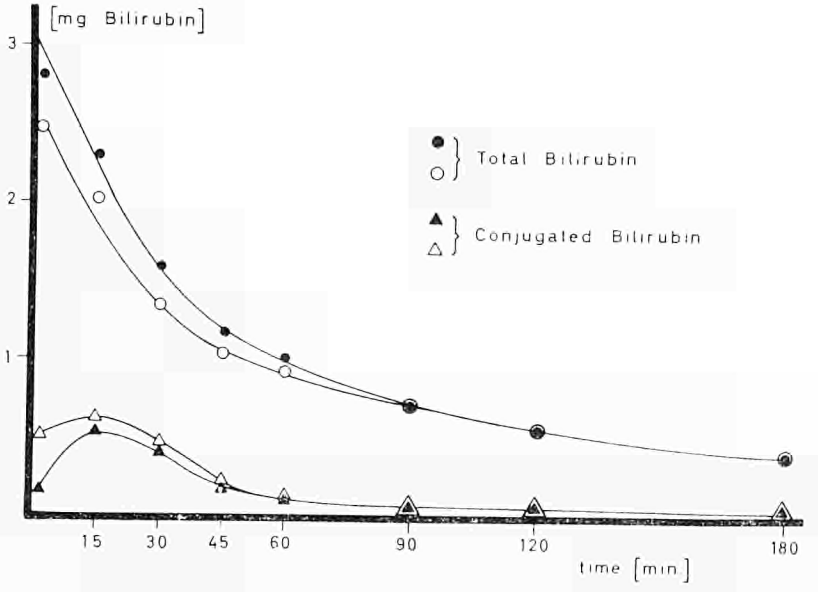

Figure 1. Total and conjugated bilirubin in the inflowing (filled signs) and outflowing (open signs) perfusate during liver-perfusion. Two samples, each of 0·7 ml, were withdrawn at the indicated points. All values are corrected for the withdrawal of samples. The conditions of the experiments were those indicated in figure 2.

Excretion of conjugated bilirubin in the bile commences after a short lag-period (figure 2), remains essentially constant for a period of 45 min, and decreases as the concentration of bilirubin in the perfusate falls to lower levels. A small proportion of bilirubin (*ca.* 20–30 per cent) is excreted as free bilirubin into the bile, but the proportion decreases during perfusion. On the other hand, the percentage of nonglucuronide and alkali-stable bilirubin remains constant.

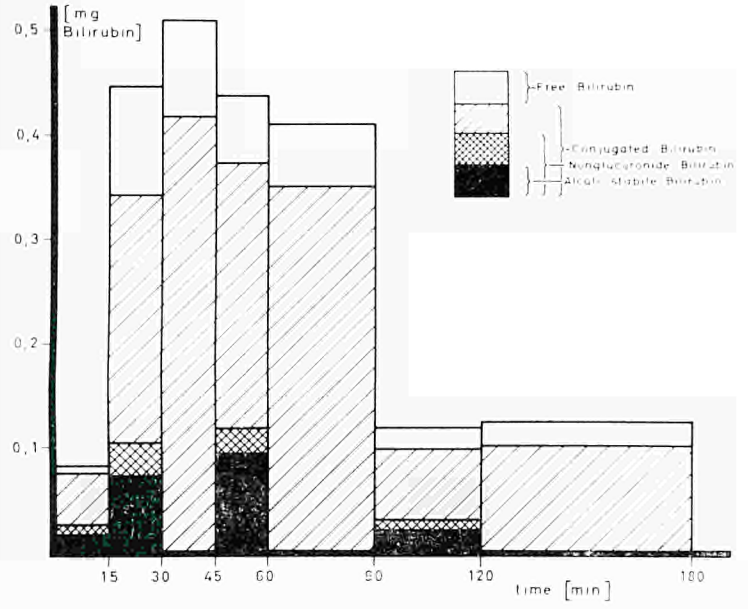

Figure 2. Excretion of free and conjugated bilirubin into the bile during perfusion of the liver. Bile was collected for 15 min intervals during the first hour, for 30 min during the second hour, and for 60 min during the third hour. 3·1 mg of bilirubin were added at time zero. The volume of the perfusate was 43 ml, the liver weight was 6·3 g. Determinations of nonglucuronide and of alkali-stable bilirubin were not carried out for the 30–45, the 60–90, and the 120–180 min collection periods.

In order to determine whether the biochemical functions of the liver remain intact during a long period of perfusion, a second dose of bilirubin was administered in one experiment. No significant difference was found between the first and the second dose with respect to removal and excretion of bilirubin. Indeed, the liver is capable of excreting much larger quantities of conjugated bilirubin (at a concentration of 15 mg per cent up to 4–5 mg hour), but the flow of bile ceases often at a later time, possibly due to crystallization of bilirubin in the bile-ducts. If the bile-duct is ligated, conjugated bilirubin is secreted into the blood, but in much smaller quantities than into the bile, and its production ceases eventually.

Rates of removal from the blood and excretion of conjugated bilirubin by normal and x-irradiated liver are shown in figure 3. Both functions are delayed after irradiation, but the pattern of removal and excretion is essentially the same as in normal liver. Average values for these rates in normal and x-irradiated liver at different periods during perfusion are presented in the

table. The rates of bilirubin-removal by x-irradiated liver are significantly lower from 15–30 min, and higher from 30–120 min, after addition of bilirubin as compared with normal liver. The rates of excretion of conjugated bilirubin into the bile by x-irradiated liver are lower during the first hour and higher

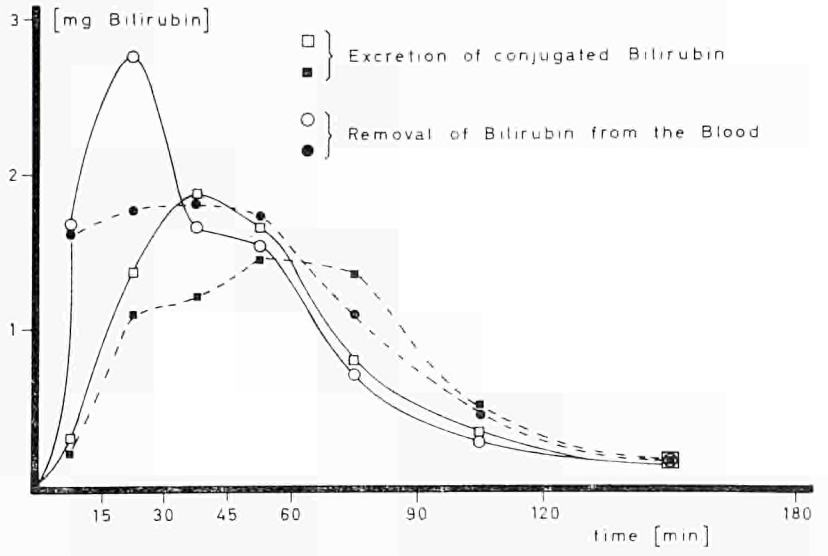

Figure 3. Rate of removal of bilirubin from the blood and rate of excretion of conjugated bilirubin into the bile during perfusion of a normal (open signs) and an x-irradiated (filled signs) liver. The values on removal were computed from the differences in concentration of the total bilirubin in the perfusate. The values on excretion were computed from the concentration of conjugated bilirubin in the bile. All rates of excretion or removal are presented in mg/hour at the intermediate point of the collection periods.

	Time (minutes)†	x-irradiated (1000 r)	Control
Rate of removal of bilirubin from the blood mg/hr/g liver	0– 15	292 ± 87‡	186 ± 90
	15– 30	348 ± 86	603 ± 91
	30– 45	325 ± 79	210 ± 75
	45– 60	313 ± 70	232 ± 70
	60–120	178 ± 27	79 ± 24
	120–180	41 ± 21	37 ± 10
Rate of excretion of conjugated bilirubin into the bile mg/hr/g liver	0– 15	59 ± 23	72 ± 21
	15– 30	172 ± 47	244 ± 55
	30– 45	194 ± 36	388 ± 126
	45– 60	234 ± 25	303 ± 85
	60– 90	236 ± 30	160 ± 25
	90–120	106 ± 29	69 ± 30
	120–180	37 ± 10	24 ± 9

† Period of collection of bile or period between two samplings from perfusate.
‡ Standard deviation of the mean.

Rate of conjugation and excretion of bilirubin by the perfused liver of normal and x-irradiated rats.

thereafter. No significant difference was found between normal and x-irradiated liver with respect to the distribution of conjugated bilirubin into glucuronide and sulphate conjugates.

Even the x-irradiated rat liver is able to conjugate and excrete considerably more bilirubin than could derive from haem and porphyrin catabolism, if bilirubin—as is the case in most mammals—were the biliary excretion product of this catabolism. It is, however, difficult to judge to what extent *in vivo* conjugation and excretion of certain other compounds, such as steroid hormones, might be altered after x-irradiation, since large differences exist in the ability of different substrates to undergo conjugation and since, therefore, the competition between substrates may result in a changed proportion between different conjugated products.

It should be pointed out, finally, that our experiments can be interpreted not only on the basis of a lowered level of conjugated enzymes in irradiated liver, but also on the basis of an alteration in the dynamics of blood-flow, or in diffusion to the enzymatic sites of x-irradiated liver.

On a étudié, dans le foie isolé et perfusé de rats normaux et irradiés, la formation de la bilirubine conjuguée et son excrétion par la bile. La bilirubine est enlevée rapidement du sang et 70–85 pour cent de cette substance est excrété par la bile aprés une période d'attente comme bilirubine conjuguée. La conjugaison se produit surtout avec l'acide glucuronique, mais également avec d'autres composés.

L'enlévement de la bilirubine du sang et l'excrétion de la bilirubine conjuguée par la bile, sont retardés dans le foie perfusé de rats irradiés.

Die Bildung konjugierten Bilirubins und seine Ausscheidung in die Galle wurde in der perfundierten Leber normaler und bestrahlter Ratten untersucht. Bilirubin wird rasch dem Blut entzogen und zu etwa 70–85 Prozent als konjugiertes Bilirubin in die Galle ausgeschieden. Die Konjugation erfolgt zum größten Teil mit Glucuronsäure, daneben auch mit anderen Verbindungen. Entfernung des Bilirubins aus dem Blut und Ausscheidung konjugierten Bilirubins in die Galle erfolgen verzögert in der perfundierten Leber bestrahlter Ratten.

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